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Zhang, Yingmiao

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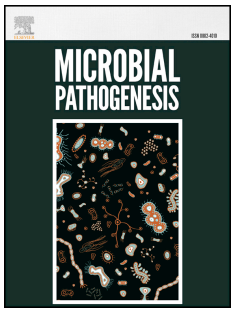

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Author statement

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Invasiveness of the *Yersinia pestis* Ail protein contributes to host dissemination in pneumonic and oral plague

Yingmiao Zhang[a], Xiaoling Ying[a,b], Yingxia He[a], Lingyu Jiang[a], Song Zhang[c], Sara Schesser Bartra[d], Gregory V. Plano[d], John D. Klena[e], Mikael Skurnik[f], Hongxiang Chen[c], Huahua Cai[a,*], Tie Chen[a,*]

[a] Department of Clinical Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Sciences and Technology, Wuhan 430030, Hubei, China.

[b] Translational Medicine Conter, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510000, Guangdong, China.

[c] Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

[d] Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL 33101, United States of America.

[e] Centers for Disease Control and Prevention, Atlanta, Georgia

[f] Department of Bacteriology and Immunology, University of Helsinki, Helsinki 00014, Finland.

***Corresponding Authors**: Tie Chen, Department of Clinical Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, China. Tel: 86-027-69378404. E-mail: chentie@hust.edu.cn or tiechen2005@yahoo.com.

Huahua Cai, Department of Clinical Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, China. E-mail: Clarelucky9@126.com.

Abstract

Yersinia pestis, a Gram-negative bacterium, is the etiologic agent of plague. A hallmark of *Y. pestis* infection is the organism's ability to rapidly disseminate through an animal host. *Y. pestis* expresses the outer membrane protein, Ail (Attachment invasion locus), which is associated with host invasion and serum resistance. However, whether Ail plays a role in host dissemination remains unclear. In this study, C57BL/6J mice were challenged with a defined *Y. pestis* strain, KimD27, or an isogenic *ail*-deleted mutant derived from KimD27 via metacarpal paw pad inoculation, nasal drops, orogastric infection, or tail vein injection to mimic bubonic, pneumonic, oral, or septicemic plague, respectively. Our results showed that *ail*-deleted *Y. pestis* KimD27 lost the ability to invade host cells, leading to failed host dissemination in the pneumonic and oral plague models but not in the bubonic or septicemic plague models, which do not require invasiveness. Therefore, this study demonstrated that whether Ail plays a role in *Y. pestis* pathogenesis depends on the infection route.

Keywords: *Yersinia pestis*; pneumonic plague; oral plague; Ail protein; invasion; host dissemination

1. Introduction

Yersinia pestis is the causative agent of bubonic and pneumonic plague and is transmitted via the bite of infected fleas or aerosols that contain the pathogen, respectively [1]. *Y. pestis* belongs to the family *Enterobacteriaceae* [2] and evolved from *Yersinia pseudotuberculosis* (*Y. pseudotuberculosis*) within the last 6,000–20,000 years [3-7]. How an ancestor of *Y. pseudotuberculosis* evolved to the remarkably different pathogen, *Y. pestis*, remains unknown. The current hypothesis is that *Y. pseudotuberculosis* acquired, deleted or mutated multiple genes during its evolution to *Y. pestis*.

Because of their ability to ferment glycerol and reduce nitrate, *Y. pestis* strains have been historically classified into the three biovars, antiqua, mediaevalis, and orientalis, which were responsible for the first, second, and third pandemics, respectively [8]. Zhou et al. recently proposed a new biovar, microtus, based on biochemical and genetic analyses [9]. Yang et al. studied the genetic history of annotated *Y. pestis* genomes and revealed an evolutionary lineage that has defined both early ancestral and modern pandemic *Y. pestis* populations based on sequential single-nucleotide polymorphism changes [5]. The *Y. pestis* strains, CO92 (biovar orientalis) and KIM (biovar mediaevalis) are modern-positioned lineages, both isolated from human plague cases [5, 10]. *Y. pestis* strain 91001 (biovar microtus) was isolated from a *Microtus*-related plague focus in China. Strain 91001 is avirulent to humans, only naturally causing plague in rodents and

their associated epizootics. The genomic structure of strain 91001 differs dramatically from those of strains CO92 and KIM because of the rearrangements mediated by insertion sequence elements, which may be responsible for human attenuation [9]. Strain KimD27, a non-pigmented isolate of Kim10 ($pCD1^+$ pgm^- $pPst^+$), is avirulent to mice [1]. During evolution, some *Y. pestis* strains, such as CO92, became the most virulent strain, while others, such as KimD27 that has lost the *pgm* locus, are conditionally virulent. The virulence of microtus strain 91001 lies between strains CO92 and KIM. Therefore, it is hypothesized that whether a *Y. pestis* strain can cause a plague depends partly on infection routes; thus, all routes of infection should be systematically studied.

The pathogenic *Yersinia* spp. share several critical virulence factors, including the virulence plasmid, pCD1/pYV, encoding a type III secretion system (T3SS) [11, 12]. The T3SS mediates cell contact-dependent injection of the *Yersinia* outer protein (Yop) effectors into targeted host cells to block bacterial phagocytosis [13] and suppress proinflammatory cytokine production [14-16]. However, the Yop-mediated effects may require *Yersinia* to adhere to host cells [17-21]. Enteropathogenic *Yersinia* expresses three dominant adhesins/invasins (YadA, invasin and Ail) that are required for efficient cell attachment and invasion [22-24]. *Y. pestis* does not express YadA or invasin [25-27], which were apparently lost during evolution; however, it does express high levels of Ail [19, 28]. In addition, *Y. pestis* acquired the plasminogen activator protease (Pla) [29] and the pH 6 antigen [30] to enhance its association with host cells.

Several experimental studies have established the roles of Ail in cell attachment/invasion, Yop injection, and serum resistance [31-35]. The essential role of

Ail as a virulence factor of *Y. pestis* has also been established in rat models of pneumonic and bubonic plague [34, 35] and mouse models of pneumonic, bubonic, and septicemic plague [19, 21, 34, 35]. However, no changes in virulence during *Y. enterocolitica* infection were observed between wild-type strains of this pathogen and its isogenic *ail*-deleted mutant [36, 37].

In this study, we systematically investigated whether Ail-mediated *Y. pestis* pathogenesis is related to the infection route during plague, by using a defined *Y. pestis* strain, KimD27, and its isogenic *ail*-deleted mutant. The results showed that Ail-mediated host invasion was required for *Y. pestis* infection and dissemination in pneumonic and oral plague but not for systematic or bubonic plague, indicating that the role of *Y. pestis* Ail in plague pathogenesis depends on the infection route.

2. Materials and Methods

2.1. Ethics statement

All animal experiments were carried out in strict accordance with the Institutional Animal Care and Use Committees and Institutional Review Board (IRB) of Tongji Hospital, Tongji Medical College, China. The mouse handling protocol and all experimental procedures were specifically approved for this study by the Medical Ethics Committee of Tongji Hospital and conducted in accordance with the institutional guidelines (IRB ID: TJ-A20141220 for animal experiments and TJ-C20140113 for human

experiments). All procedures on mice were performed under anesthesia. All volunteers (serum donors) involved in the experiment signed consent forms.

2.2. Mice

C57BL/6 wild-type mice were purchased from Wuhan University Animal Center, Wuhan, China. All mice were housed in pathogen-free conditions and treated in direct accordance with guidelines drafted by the Animal Care Committees of Tongji Hospital.

2.3. Bacterial strains and plasmids

Y. pestis strain KimD27 is a non-pigmented isolate of Kim10 ($pCD1^+$ pgm^- $pPst^+$) [1]. *Y. pestis* strain 1418 used in this study originated from KimD27 [38]. *Y. pestis* 91001 (biovar microtus) was isolated from a *Microtus*-related plague focus in China and is avirulent to humans, only naturally causing plague in small rodents [9]. The *Yersinia* strains were cultured on GC-based plates (Difco, Sparks, MD, USA) supplemented with 1% hemoglobin (USB Co., Cleveland, OH, USA). All strains of *Yersinia* spp. used in this study were cultured at 26°C [39].

pCVD442-*ail*::KmGB is a suicide vector, carrying an *ail* gene-knockout sequence with ampicillin and kanamycin resistance and stored in *E. coli* S17-1λpir [40]. pSE380-*ail* is an expression plasmid that expresses the *ail* gene of *Y. pestis* [41]. The pXEN-*lux*CDABE (pXEN-18) plasmid, a gift from Dr. Ruifu Yang, Beijing Institute of Microbiology and Epidemiology, Beijing, China, contains a *lux* gene that can generate luminescence in *Yersinia spp*. [42].

2.4. Construction of ail-knockout and knockin Y. pestis strains

The suicide plasmid pCVD442-ail::KmGB was mobilized into *Y. pestis* strain KimD27, as previously described by Ho et al. [43]. In brief, the suicide vector presented in *E. coli* S17-1λpir was introduced into *Y. pestis* KimD27 via a typical conjugation assay. Kanamycin-resistant transconjugants were selected using the *Yersinia*-selective agar plate (BD, Franklin Lakes, NJ, USA), for counter-selection of donors. Selected transconjugants were plated onto Luria-Bertani (LB) agar with 10% sucrose (Sigma-Aldrich, St. Louis, MO, USA) and cultured at ambient temperature for 2 days. Correct allelic exchange in the resulting Suc^r Kan^r colonies was confirmed using PCR with the corresponding primers: ATGGTTTTTATGAATAAGATATTACTGGTC/TTAGAACCGGTAACCCGC. The plasmid pSE380-*ail* was transformed into the *ail*-knockout strain, *Y. pestis* KimD27 Ail(-) to obtain the *ail*-complemented knockin strain. The *virF* gene (primers: TCATGGCAGAAC/AGCAGTCAG/ACTCATCTTACCATTAAGAAG) on the pYV plasmid was used as a positive control [44]. The construction of *ail*-knockout and knockin *Y. pestis* 91001 followed the same methods described above.

2.5. Bioluminescent Y. pestis KimD27

Y. pestis KimD27 and *Y. pestis* KimD27 Ail(-) were transformed with pXEN-18 by electroporation. *Y. pestis* strains with the pXEN-18 plasmid generate luminescence that can be detected by the Night OWL II LB983 imaging system (Berthold Technologies, Bad Wildbad, Germany) [42]. The plasmid was identified via PCR (primers: TCTCAAACAGAGGTAATGAAACG/ CATCAAAAATAGTCGTAGCAT) [42].

2.6. Serum-killing assay

Bacterial resistance to complement in fresh serum from C57BL/6J mice and human volunteers was determined as described previously [31, 45]. Animal serum was collected from C57BL/6 mice by heart puncture after anesthesia. Clotted blood samples were centrifuged at 1000 ×*g* for 15 min to obtain serum. Heat-inactivated serum was used as a control after heating at 56°C for 30 min. Fresh LB media were inoculated separately with *Y. pestis* strains. After culturing for 18 h, the bacteria were suspended in sterilized phosphate-buffered saline (PBS) to an OD_{600} of 0.2 and diluted to 1:1000 in PBS. The diluted bacterial suspension (50μl) was added to 200μl of normal human serum (NHS) and normal mouse serum. The samples were incubated at 37°C in 5% CO_2 for 60 min. The mixtures were serially diluted 10-fold, and the viable bacteria were counted via LB agar plating. Serum resistance levels were determined by comparing the number of surviving bacteria (colony-forming units; CFUs) treated with fresh NHS to the number of the surviving bacteria treated with heat-inactivated NHS (defined as 100%).

2.7. Cell invasion assay

The cell invasion assay has been described previously [46]. Briefly, Chinese hamster ovary (CHO) cells were cultured in RPMI-1640 medium (Life Technology, Grand Island, NY, USA) with 2% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1×10^5/ml in 24-well plates (BD, Franklin Lakes, NJ, USA). After adding 1 ml of bacterial suspension without FCS at 1×10^7 CFU/ml, cells were incubated for 2.5 h at 37°C in 5% CO_2. To determine the bacterial internalization, gentamicin (Invitrogen, Carlsbad, CA, USA), which kills extracellular bacteria but cannot penetrate host cells, was added to each well to a final concentration of 100 μg/ml, and the cultures were

171 incubated for 60 min. Cells were washed three times to remove the antibiotic, then
172 suspended in PBS containing 0.5% saponin, diluted, and plated on both the LB and *Y.*
173 *pestis* plates. The bacterial internalization levels were determined by counting CFUs
174 recovered from lysed cells. All experiments were performed in triplicate, and the data are
175 expressed as the means ± standard error of mean.

176 *2.8. Animal challenge for infection and dissemination*

177 To confirm whether Ail plays a role in plague *in vivo*, mice were inoculated either
178 subcutaneously, intranasally, orogastrically or intravenously via hind paw injection, nasal
179 dropping, orogastric infection via catheter, or tail vein injection, respectively. Three
180 separate experiments were conducted, including dissemination by CFU counting,
181 dissemination by *in vivo* imaging, and infectivity by survival rate. The dissemination rate
182 was defined as the transport of *Y. pestis* to the lymph nodes, livers, spleens and lungs [47].
183 The infectivity was defined as the mortality after pathogen inoculation. All the mice were
184 active without adverse state performance. No mice died before meeting the criteria for
185 euthanasia.

186 *2.8.1 Dissemination*

187 C57BL/6J mice were infected with *Y. pestis* KimD27 and *Y. pestis* KimD27 Ail(-)
188 via subcutaneous injection (1×10^8 CFUs), nasal drops (5×10^7 CFUs), orogastric
189 infection (4×10^8 CFUs) or intravenous injection (1×10^5 CFUs) after anesthesia (orogastric
190 infection was without anesthesia) to mimic bubonic, pneumonic, oral or septicemic
191 plague, respectively. We sacrificed the mice that underwent subcutaneous, intravenous

and intranasal injections at 48 hours post-inoculation and the mice that underwent orogastric infection at 72 hours post-inoculation. At the indicated time points, the mice were euthanized, and their inguinal lymph nodes, spleens, livers and lungs were collected aseptically, weighed and homogenized in sterilized PBS. The homogenized organs were then treated with 1% Triton X-100 (Biosharp, Shenzhen, China) for 10 min to release the bacteria, and serial dilutions were plated for CFU counts.

2.8.2 In vivo imaging

C57BL/6J mice were anesthetized with 2% isoflurane for the entire one-time imaging process in an isolation chamber using an IVIS Spectrum instrument (Caliper, Shanghai, China). Mice were imaged at 0 and 48 or 72 hours post-infection (48 hours for subcutaneous, intravenous and intranasal infection, 72 hours for orogastric infection). Radiance signaling was measured in photons/sec/cm^2/steradian and analyzed using Living Image Software V.4.2 (Caliper) as described previously [48]. To better show the luminous signals in the infected area on the imaging system, the chests and abdomens of mice were surgically opened.

2.8.3 Survival analysis

The mice were inoculated with *Y. pestis* KimD27 or *Y. pestis* KimD27 Ail(-) in a similar manner to that described for the *in vivo* dissemination assay for the different infection routes. For *Y. pestis* 91001 and its derivatives, mice (n=8/group) were intravenously inoculated with 300 CFUs of 91001 or 91001 Ail(-). The survival rates of

the mice were recorded every 12 hours up to 14 days post-infection. Mice were euthanized upon reaching humane endpoints.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 6.0 (GraphPad, San Diego, CA, USA). Data are presented as the mean values ± standard error of mean (SEM). The difference between two groups was determined using a two-tailed Student's *t*-test. The survival rates in the different infection groups were compared with a log-rank test using Kaplan-Meier analysis. A probability value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Construction of ail-knockout Y. pestis KimD27

The *ail*-knockout *Y. pestis* KimD27 was constructed using a suicide plasmid and subsequent selection methods. The *ail*-knockout strain and complementary knockin strain were confirmed by PCR for detecting the virulent plasmid and *ail* gene in the *Y. pestis* strain KimD27 (Fig. 1A).

3.2. Knocking out ail in Y. pestis KimD27 decreased the bacterial ability to invade epithelial cells and conferred sensitivity to being killed by human serum

To confirm the functional deletion of *ail*, *Y. pestis* KimD27 and *Y. pestis* KimD27 Ail(-) were first examined for serum sensitivity. Similar to the results of previous studies [28, 31, 32, 35], the *ail* mutant became sensitized to normal human serum but not to mouse serum [31] (Fig. 1B). *Y. pestis* KimD27 Ail(-) was also tested for its ability to invade CHO cells. Fig. 1C shows that the ability of *Y. pestis* KimD27 Ail(-) to invade host cells was significantly reduced. However, both the serum resistance and invasion ability were recovered in the complementary *ail*-knockin strain.

3.3. Y. pestis KimD27 Ail(-) lost its dissemination ability when challenged intranasally and orogastrically

The bacterial dissemination was determined by counting the bacterial loads in individual organs (lymph node, spleen, liver and lungs; Fig. 2A) and tracing the fluorescence intensity of the bioluminescence (Fig. 2B) with the pXEN-18 plasmid-containing *Y. pestis*. Strain KimD27 Ail(-) showed significantly reduced bacterial loads compared with those of the WT strain upon intranasal and orogastric infection but not upon subcutaneous or intravenous infection (Fig. 2A). The *in vivo* imaging results were essentially the same to those observed for the bacterial loads in the organs (Fig. 2B), indicating that Ail is essential for *Y. pestis* KimD27 host dissemination in pneumonic and oral plague.

3.4. Y. pestis KimD27 Ail(-) did not cause pneumonic or oral plague

Y. pestis KimD27 caused typical infections through all infection routes, although the inoculation concentrations were much higher than those typically used for fully virulent wild-type *Y. pestis* (Fig. 3) [19, 47, 49]. Upon losing Ail expression, *Y. pestis* KimD27 completely lost the ability to cause pneumonic and intragastric infections at the highest inoculation level used. Moreover, the *ail* mutant maintained a reduced capacity to induce bubonic and septicemic plagues. These results evidence that different infection routes can lead to diverse infection outcomes for *Y. pestis.*

3.5 Ail plays roles in host cell invasion and human serum sensitivity in Y. pestis 91001

Using surrogate strains that are less restricted by regulatory burdens imposed by the National Select Agent Registry or equivalent rules can add great value to a study; however, authors of studies using such strains (particularly in animal models) must justify why the resulting data are relevant to the biology of the wild-type strain. We therefore evaluated the invasion and serum resistance of *Y. pestis* 91001 and its *ail* mutant derivatives. The *ail* mutant became sensitized to normal human serum but not to mouse serum (Fig. 4A). The invasion of 91001 Ail(-) to CHO cells was significantly reduced compared to the 91001 or 91001 Ail(-) with *ail* complement (Fig. 4B). Moreover, mice were inoculated though tail vein injection with 91001 and 91001 Ail(-), and there were no differences in survival rate of mice infected with these two strains (Fig. 4C). This result is consistent with the observation from KimD27. The microtus strain 91001 between modern lineages and *Y. pseudotuberculosis* is thought to be an intermediate, from which

all modern plague strains have evolved. Thus, this result indicates that Ail plays roles in invasion and serum resistance in different lineages of *Y. pestis*.

4. Discussion

To cause plague, *Y. pestis* must overcome the host's first lines of defense, such as the skin and mucosal surfaces. Current studies suggest that the role of *Y. pestis* Ail in developing plague results from its ability to promote bacterial invasion in murine hosts. In this study, we used a single KIM strain to mimic four plague types and systematically investigated *Y. pestis* Ail's role in plague pathogenesis. We demonstrated that Ail plays an essential role in initiating host infection and dissemination during pneumonic and oral plague, and the role of *Y. pestis* Ail in this pathogenesis is related to the infection route. Moreover, using two *Y. pestis* strains, an attenuated strain and wild-type (fully virulent) strain, we concluded that *Y. pestis* strains from different lineages might yield different results.

Our results are in accordance with the conclusion that Ail plays a significant role in pneumonic and oral plague but not in bubonic or septicemic plague. However, studies by Felek et al. showed that Ail is essential for virulence in the intravenous route in a KIM5 (pgm^-) strains [19], which differs from that reported in this study (Fig. 3C and Fig. 4C). It is unclear whether the alternative method for *ail* deletion has effect on the virulence of mutant or whether the discrepancies in our results are due to different strains of mice. It should be noted that Felek et al. also showed that although the calculated LD_{50} of the

KIM5 Δ*ail* mutant is much higher than that of KIM5, 100 organisms can kill a few mice in some cases, suggesting that the expression of virulence factors of *Y. pestis* may be very sensitive to the environment. Additionally, Bartra et al. showed that Ail is not required for virulence in an intravenous mouse model of plague, by using retro-orbital inoculation with *Y. pestis* KIM5 [31]. In fact, the reduced role of Ail in septicemic plague (maybe in other routes) is likely due in part to the lack of bactericidal activity of mouse complement; thus, the role of Ail in serum resistance is not as important in mice. Unlike with the mechanistic entries that *Y. pestis* uses to cause bubonic and septicemic plague, we speculate that after entering the lungs or digestive system via aspiration or feeding, *Y. pestis* uses its Ail protein [31] and plasminogen activator (Pla) [50] in addition to other factors to bind to and invade the mucosal layers in the bronchial and digestive tracts. *Y. pestis* may then hijack antigen-presenting cells, such as macrophages or dendritic cells, to promote host dissemination.

The results presented herein may help explain discrepancies in the data presented in previous studies. Ail plays an important role in plague in rat and mouse models [19, 34, 35]. However, the virulence was unchanged between the wild-type strain and its isogenic *ail*-deleted mutant during *Y. enterocolitica* infection [36, 37]. *Y. enterocolitica* and *Y. pseudotuberculosis* express all three invasion-related genes (YadA, invasin and Ail). Invasin has shown to induce the strongest bacterial invasion into epithelial cells, especially when bacteria are grown at 26°C [51]. Krukonis et al. demonstrated that the Ail protein in *Y. pseudotuberculosis* YPIII had significantly decreased adhesive and invasive abilities compared with those of *Y. pestis*. We therefore speculate that unlike Ail in *Y.*

316 *pestis*, Ail may not be essential for *Y. enterocolitica* or *Y. pseudotuberculosis* to invade
317 hosts.

318 *Y. pestis* strain CO92 and KIM are modern-positioned lineage strains both isolated
319 from human plague cases. Using a fully virulent CO92 strain and its derivatives,
320 Kolodziejek and colleagues demonstrated that *Y. pestis* Ail contributes to the virulence of
321 pneumonic plague [35] and protects against complement-mediated lysis in bubonic
322 plague pathogenesis in mice and rats [34]. However, the LD_{50} of certain *Y. pestis* strains,
323 such as CO92, has been reported to be as low as one CFU in a murine model [52, 53].
324 Notably, the KIM strains are naturally attenuated due to the loss of the pgm locus, and
325 studies with such strains may represent another example of Ail's role in the *Y. pestis*
326 pathogenesis. We therefore believe that the attenuated strain may have some advantages
327 over the fully virulent strains for studying host-pathogen interactions. Our recent work
328 demonstrated that *Y. pestis* interacts with SIGNR1 (CD209b), a C-type lectin receptor on
329 antigen-presenting cells, leading to bacterial infection and dissemination [39] using *Y.*
330 *pestis* strain 1418. This strain originated from KimD27, but its 104-kb pigmentation locus
331 has been deleted [38], and it is therefore classified as an avirulent and a nonselect agent
332 strain. To further address Ail' role in *Y. pestis*, we examined serum sensitivity as well as
333 invasion of *Y. pestis* 91001 and its Δ*ail* derivatives, the result indicates Ail plays roles in
334 in different lineages of *Y. pestis.*

335 In a reported fatal laboratory-acquired infection case, a 60-year-old researcher at the
336 University of Chicago died of infection from the attenuated *Y. pestis* strain, KimD27 [54].
337 Because this strain was excluded from the National Select Agent Registry and was not

known to have caused laboratory-acquired infections or human fatalities, this researcher became infected with the strain in a biosafety level II setting. Postmortem examination revealed that the researcher had hereditary hemochromatosis, which is an iron-overload disease, with increased iron absorption and storage in multiple organs [54, 55]. *Y. pestis* infection needs iron [56-58], and the infection described here likely occurred because hemochromatosis-induced iron overload enabled the *Y. pestis* strain KimD27, which lacks the pgm locus that includes a high-affinity iron transport system, to infect this researcher. The conclusion from this incident was that the ability of the attenuated *Y. pestis* strain KimD27 to cause plague depends on the specific circumstances. In our study, we investigated four routes by which *Y. pestis* KimD27 can cause infection and found that the Ail-mediated pathogenesis of *Y. pestis* KimD27 depended on the infection route. This finding has been previously unreported; therefore, we believe that this is a novelty of our study.

Different laboratories have used various strains to establish the pathogenic roles of Ail as an essential virulence factor of *Y. pestis* in rat models of pneumonic and bubonic plague [34, 35] and in mouse models of pneumonic, bubonic, and septicemic plague [19, 21, 34, 35]. Because of restrictions imposed by local and federal regulations for using this "select agent", we obtained *in vivo* imaging data that would have been impossible to produce if a fully virulent strain had been used. Thus, our results show that the Ail protein of *Y. pestis* plays a role in initiating host infection and dissemination during pneumonic and oral plague, and the role of Ail in *Y. pestis* pathogenesis depends on the infection route.

Competing interests

The authors declare no competing interests.

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Author contributions

Yingmiao Zhang: Methodology, Software, Investigation, Writing-Original Draft. **Xiaoling Ying:** Methodology, Investigation, Formal analysis. **Yingxia He:** Conceptualization, Resources, Visualization. **Lingyu Jiang:** Methodology, Resources. **Song Zhang:** Investigation, Software. **Sara Schesser Bartra;** Conceptualization, Writing-Review &

Editing. **Gregory V. Plano:** Conceptualization, Writing-Review & Editing. **John D. Klena:** Writing-Review & Editing, **Mikael Skurnik:** Conceptualization, Resources. **Hongxiang Chen:** Writing-Review & Editing. **Huahua Cai:** Resources, Writing-Original Draft. **Tie Chen:** Project administration, Funding acquisition.

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542

543 Figure legends

544 **Fig. 1. *Y. pestis* KimD27 Ail(-) could not invade epithelial cells or confer resistance to**
545 **bacterial killing via serum in humans.** Deletion of *ail* was confirmed via PCR, serum
546 resistance testing and cell invasion assays. (A) *Y. pestis* KimD27 Ail(-) yielded a PCR
547 product of a virulent plasmid pYV, but no product of *ail*. (B) *Y. pestis* KimD27 Ail(-) lost
548 its resistance to being killed by normal human serum but not mouse serum. (C) *Y. pestis*
549 KimD27 Ail(-) showed a decreased ability to invade epithelial cells. The results presented
550 here were obtained from three independent experiments and analyzed by Student's *t*-test
551 as the mean ± SEM (**p<0.01, ***p<0.001).

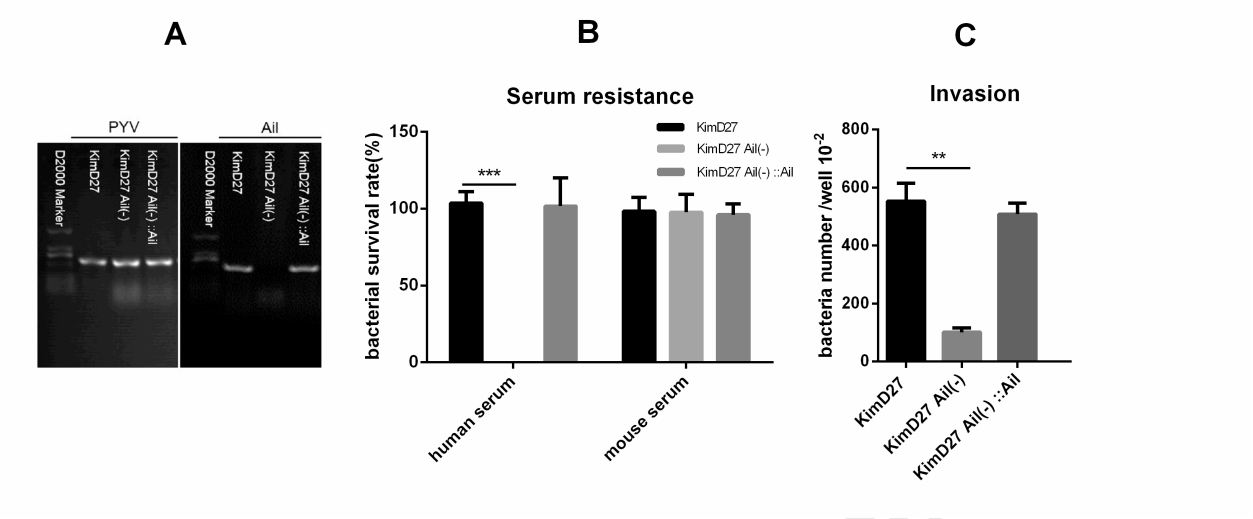
552 **Fig. 2. *Y. pestis* KimD27 Ail(-) exhibited attenuated ability to be disseminated in mice.**
553 (A) Bacterial loads in organs of infected mice (n=8/group). Mice inoculated intravenously,

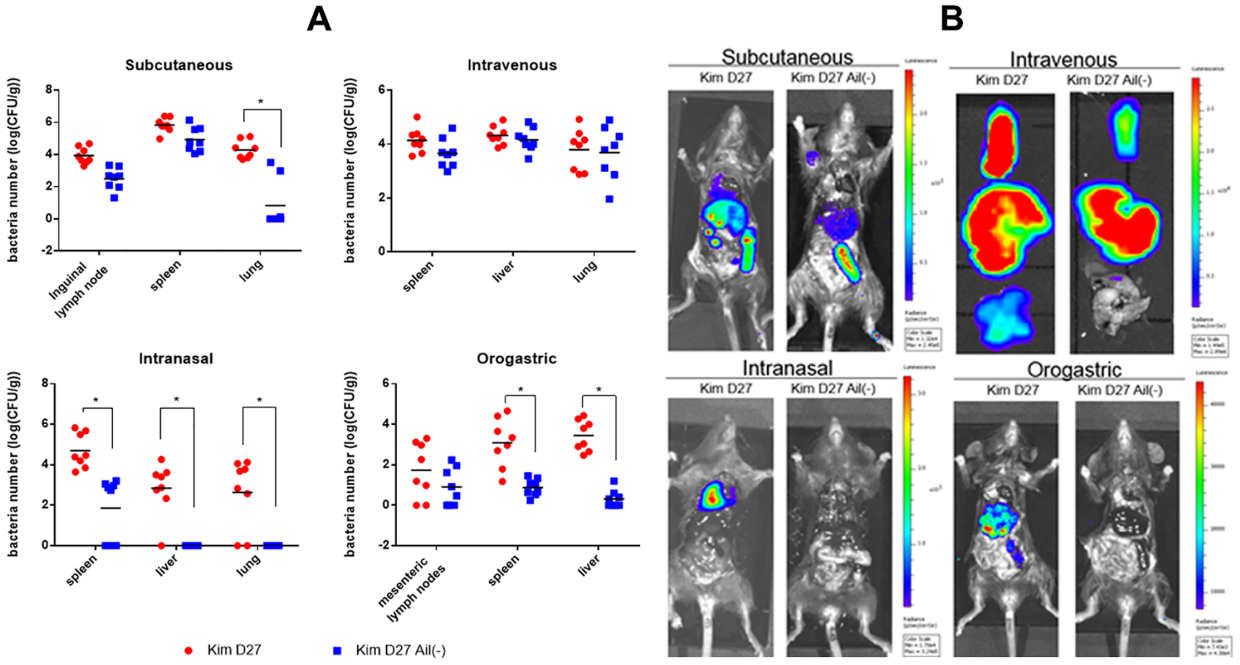
554 subcutaneously and intranasally were euthanized on day 2; mice inoculated orogastrically
555 were euthanized on day 3. The spleens, livers and lungs were removed, weighed,
556 homogenized and spread onto LB plates. The dissemination rate was determined by
557 counting the CFUs recovered from the whole lymph nodes, spleens, livers and lungs.
558 Colonization results were statistically analyzed using a two-tailed, two-sample Wilcoxon
559 rank-sum (Mann-Whitney) test (*$p<0.05$). (B) Bioluminescent imaging of lymph nodes,
560 spleens, livers and lungs of the mice infected with *Y. pestis* KimD27 pXEN-18 and *Y.*
561 *pestis* KimD27 Ail(-) pXEN-18 (n=3/group). The bioluminescent scale ranges from most
562 intense (red) to least intense (violet). To better show the infected areas of the lung, spleen
563 and liver on the imaging system, the organs were removed from the intravenously
564 infected mice. For the subcutaneously, intranasally and orogastrically infected mice, the
565 chests and abdomens were opened.

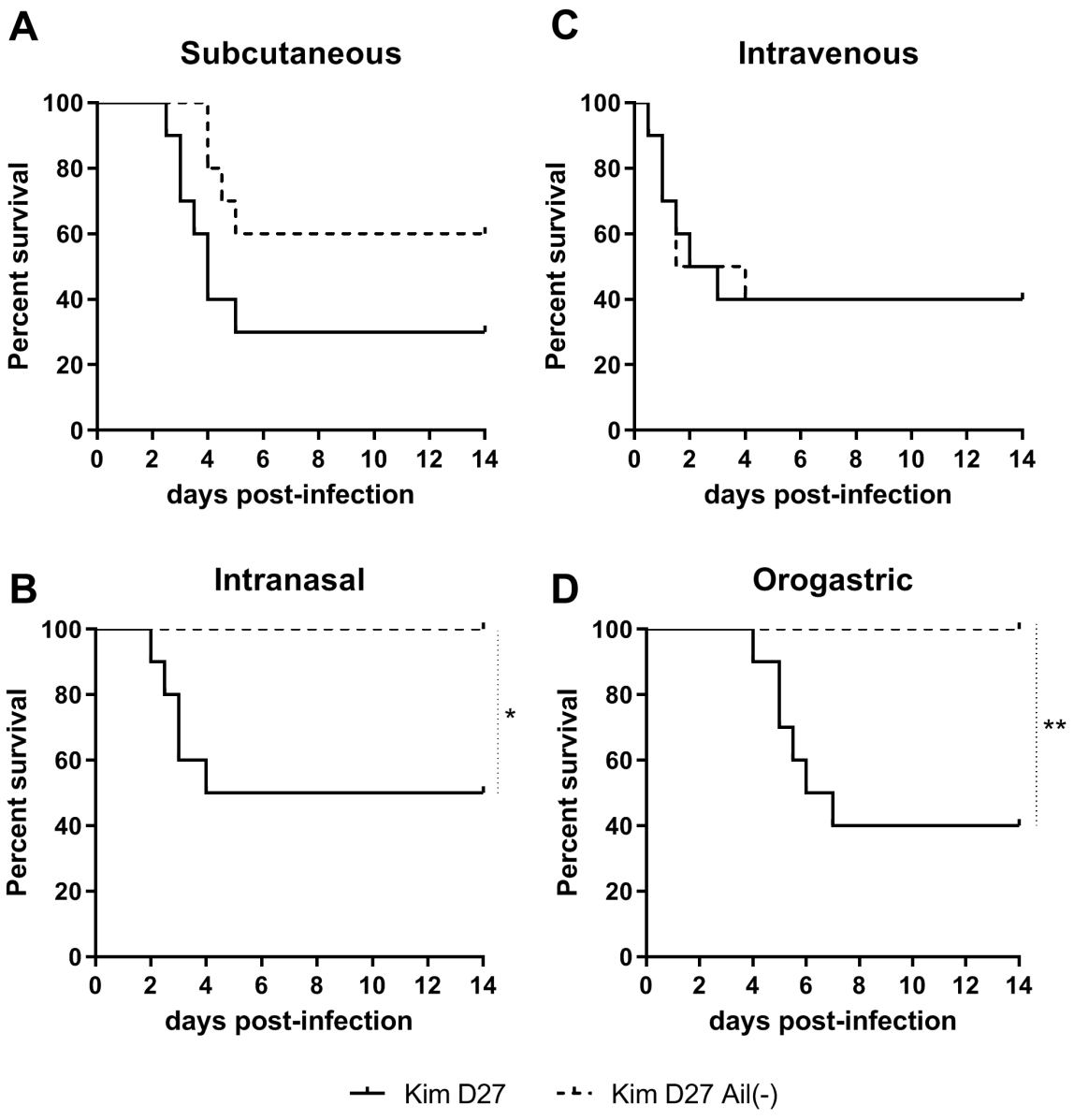
566 **Fig. 3. *Y. pestis* KimD27 Ail(-) did not cause pneumonic or oral plague but induced**
567 **bubonic and septicemic plague at reduced levels in mice.** Mice were infected with *Y.*
568 *pestis* KimD27 and *Y. pestis* KimD27 Ail(-) by (A) metacarpal paw pad injection, (B)
569 nasal drops, (C) tail vein injection or (D) orogastric infection to mimic bubonic,
570 pneumonic, septicemic, or oral plague, respectively. The data presented were pooled from
571 three independent experiments. The survival rates were compared via log-rank test using
572 Kaplan-Meier analysis (*$p<0.05$, **<0.01).

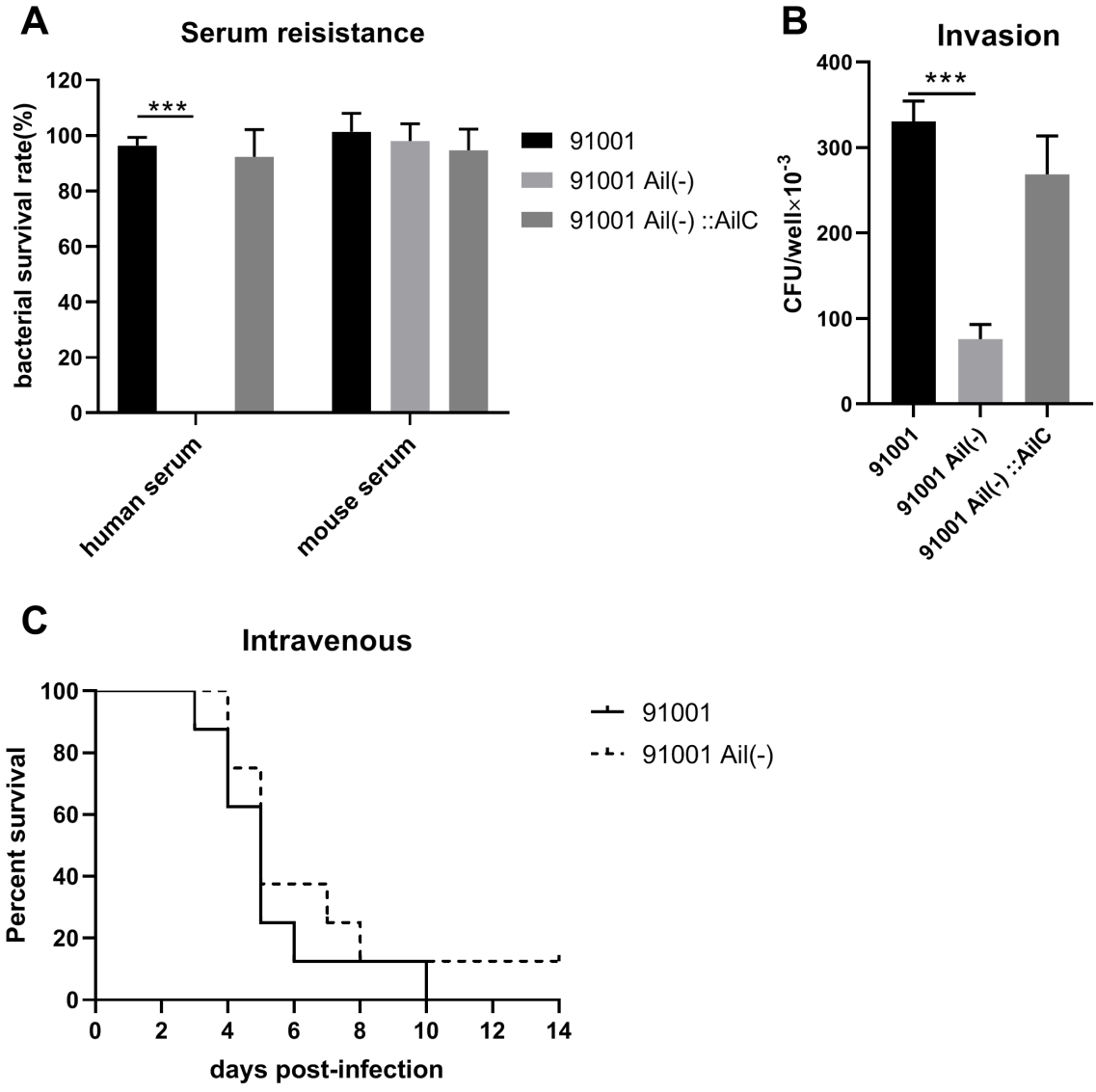
573 **Fig. 4. Ail confers resistance to bacterial killing by human serum and promotes**
574 **invasion in *Y. pestis* 91001.** (A) Serum sensitivity and (B) invasion of *Y. pestis* 91001
575 and its Δ*ail* derivatives were tested as described in Methods section. The data presented

were pooled from three independent experiments and analyzed by Student's *t*-test as the mean ± SEM (***p<0.001). (C) Mice were intravenously inoculated with strain 91001 and 91001 Ail(-), and survival rates were compared via log-rank test using Kaplan-Meier analysis.






A
Subcutaneous
Percent survival
days post-infection
C
Intravenous
Percent survival
days post-infection
B
Intranasal
Percent survival
days post-infection
*
D
Orogastric
Percent survival
days post-infection
**
Kim D27
Kim D27 Ail(-)


A
Serum reisistance
bacterial survival rate(%)
120
100
80
60
40
20
0
***
human serum
mouse serum
91001
91001 Ail(-)
91001 Ail(-) ::AilC
B
Invasion
CFU/well×10-3
400
300
200
100
0
***
91001
91001 Ail(-)
91001 Ail(-) ::AilC
C
Intravenous
Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
days post-infection
91001
91001 Ail(-)

Yersinia pestis Ail protein promotes host dissemination in pneumonic and oral plague.

The role of *Yersinia pestis* Ail protein in pathogenesis depends on infection routes.

Ail plays roles in invasion and serum resistance in different lineages of *Y. pestis*